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Distinct Intracellular Signaling in Tumor Necrosis Factor-related Apoptosis-inducing Ligand- and CD95 Ligand-mediated Apoptosis*

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent inducer of apoptosis in tumor cells but not in healthy cells. Similar to CD95 ligand (CD95L), TRAIL signaling requires ligand-receptor interaction; the downstream signaling molecules, such as Fas-associated death domain and caspase-8, also seem similar. Using cells stably expressing TRAIL and CD95L, we show that both TRAIL and CD95L induce apoptosis in the rat colon carcinoma cell line CC531. The mitochondrial damage (loss of mitochondrial membrane potential (MMP) and release of cytochrome *c*) observed after co-incubation with TRAIL-expressing cells occurs much earlier than that observed with CD95L-expressing cells. The decrease in MMP induced by both ligands was caspase-8-mediated; no difference in caspase-8 activation by TRAIL and CD95L was found. TRAIL, but not CD95L, induced activation of caspase-10. bcl-2 overexpression could not prevent TRAIL-induced mitochondrial dysfunction, whereas it completely prevented CD95L-mediated loss of MMP and cytochrome *c* release. The selective effect of TRAIL on tumor cells and the apparent inability of bcl-2 to block TRAIL-induced apoptosis suggest that TRAIL may offer a lead for cancer therapy in the future.

Cytotoxic lymphocytes employ two different strategies to kill their targets: the granule secretory pathway and the ligand-receptor interaction pathway. Ligands include CD95L[1] (Fas ligand/Apo-1 ligand) (1, 2), TRAIL (3, 4), tumor necrosis factor α (5–7), and tumor necrosis factor-related weak inducer of apoptosis (Apo-3 ligand) (8, 9), forming the tumor necrosis factor superfamily (3, 4, 10, 11). Recombinant CD95L and TRAIL kill implanted tumor cells after injection in test animals (12–14). However, CD95L has severe toxic side effects, predominantly in the liver (15, 16). In contrast, TRAIL appears to be much less toxic (12, 13), whereas it is as efficient as CD95L in killing tumor cells. Therefore, TRAIL seems more suitable for therapeutic strategies directed against tumor cells than CD95L.

CD95L and TRAIL bind to different receptors on the plasma membrane of target cells. CD95L binds to CD95, which via the adapter molecule Fas-associated death domain activates caspase-8 (17–19). TRAIL can bind to DR4 (20) and DR5 (21), which are also coupled to caspase-8 (and possibly caspase-10) (22); alternatively, it can bind to the decoy receptors DcR1 (23) and DcR2 (24), which have no death domain or a truncated death domain, respectively.

The reason for the striking difference in toxicity after *in vivo* application could be a difference in expression of the receptors for both ligands by tumor cells as compared with nontransformed cells and/or a different intracellular signaling route after binding to the receptor. It has been described that depending on the cell type, binding of CD95L can activate two routes downstream of caspase-8 activation: type I apoptosis signaling (direct activation of effector caspases by caspase-8 (18)) and type II apoptosis (cleavage of proapoptotic members of the bcl-2 family, loss of mitochondrial membrane potential (25, 26), release of cytochrome *c* (27), and activation of caspase-9 (28, 29)). The routes downstream of TRAIL binding are less well known. Thomas *et al.* (30) noted that TRAIL induces a decrease in the mitochondrial membrane potential ($\Delta\Psi$m) in human melanoma cells, whereas CD95L had no effect on the $\Delta\Psi$m in these cells. Apparently, TRAIL induces type II apoptosis. A similar finding was reported by Kim *et al.* (10) using Jurkat, U937, and MCF-7 cells. If TRAIL predominantly induces type II apoptosis, it can be expected to be inhibited by bcl-2 overexpression. However, reports are conflicting. Thomas *et al.* (30) reported total protection against TRAIL-induced apoptosis, whereas Kim *et al.* (10) found that overexpression of bcl-2 delayed but did not block apoptosis.

A systematic comparison of the intracellular routes after CD95L or TRAIL signaling has, to our knowledge, never been published. Because the reason for the difference in *in vivo* application could also be due to intracellular differences, we performed the present study, using CC531 cells as targets; this cell type expresses CD95 and all four TRAIL receptors. Rather than using recombinant proteins, we used 2PK3 cells stably expressing either CD95L or TRAIL to induce apoptosis because it was reported that membrane-expressed CD95L was more efficient (31). We show that TRAIL causes a much more potent dysfunction of the mitochondria than CD95L, whereas the extent of apoptosis was comparable. This suggests that CD95L mainly caused type I apoptosis, whereas TRAIL induced type II apoptosis. Inhibition of caspase-8 was sufficient to prevent mitochondrial dysfunction in both cases. We found a striking difference of overexpression of bcl-2 on apoptosis induction by TRAIL and CD95L: bcl-2 could not prevent TRAIL-induced mitochondrial dysfunction, whereas it completely blocked the effect of CD95L. Also, in the bcl-2-overexpressing cells, mito-

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[1] The abbreviations used are: CD95L, CD95 ligand; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-IEPD-CHO, acetyl-Ile-Glu-Pro-Asp-aldehyde; FLIP, Flice inhibitory protein; mCD95L, murine CD95L; PI, propidium iodide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; mTRAIL, murine TRAIL; zVAD-fmk, z-Val-Ala-DL-Asp-fluormethylketone; DR, death receptor; DcR, decoy receptor; BB, binding buffer.

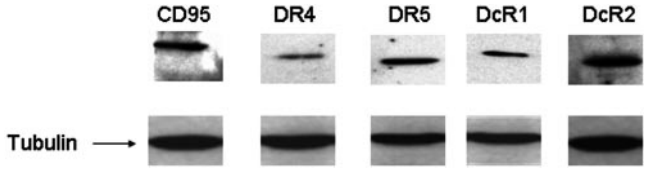

FIG. 1. **Presence of CD95 and four TRAIL receptors in CC531s cells.** CC531s cells were cultured, lysed, and immunoblotted for CD95, DR4, DR5, DcR1, and DcR2.

chondrial dysfunction was a caspase-8-mediated effect. We conclude that TRAIL and CD95L signaling trigger distinct intracellular routes in CC531 cells.

EXPERIMENTAL PROCEDURES

Reagents—The caspase inhibitors zVAD-fmk, Ac-DEVD-CHO, and Ac-IEPD-CHO were from Bachem (Bubendorf, Switzerland). Molecular Probes (Leiden, The Netherlands) supplied calcein-AM, and propidium iodide was purchased from Sigma. Annexin V was purchased from Roche Molecular Biochemicals and labeled with allophycocyanin, a 652 nm fluorescent probe (Prozyme).

Antibodies—Anti-CD95, anti-DR4, and anti-bcl-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-DR5 was obtained from Stressgen Biotechnologies (Victoria, Canada), and anti-DcR1 and anti-DcR2 were obtained from Prosci Inc. (San Diego, CA). The antibody against cytochrome *c* was purchased from BD PharMingen. Dr. J. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands) kindly provided antibodies against FLIP and caspase-8. All horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson Laboratories (West Grove, PA).

Cell Lines—CC531 cells were cultured in RPMI 1640 "Dutch modification" medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 4 mM L-glutamine (Invitrogen). The bcl-2 transfectants, a gift from Rob Hoetelmans (Department of Surgery, LUMC, Leiden, The Netherlands) were cultured as described previously (32).

Mouse B-cell lymphoma 2PK3-mock, 2PK3-CD95L, and 2PK3-mTRAIL cells (a kind gift from Hideo Yagita; Tokyo, Japan) were cultured in RPMI 1640 medium (L-glutamine, 25 mM HEPES) (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin.

Co-incubation of 2PK3 Cells and CC531 Cells—CC531 cells and 2PK3 cells were harvested and washed twice. The CC531 cells were labeled by incubation with 1 μM calcein-AM for 15 min in culture medium. Subsequently, these cells were washed and seeded in a 96-well U-bottomed plate at a density of 1×10^5 cells/50 μl/well. One million 2PK3 cells were added in a volume of 100 μl. After the indicated time period, the 96-well plate was centrifuged for 3 min at 1500 rpm at 4 °C. The supernatant was removed, the cells were washed with 100 μl of phosphate-buffered saline, and the detached cells were transferred to a clean plate. Next, the original plate was trypsinized for 10 min at 37 °C, and the cells were added to the phosphate-buffered saline fraction. The plate was centrifuged again for 3 min at $500 \times g$ at 4 °C. The cells were suspended in 200 μl of binding buffer (BB) containing 0.5 μl of annexin V (0.5 μg/μl) for flow cytometric analysis. The BB consisted of 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.0 mM $MgCl_2 \cdot 6H_2O$, and 1.8 mM $CaCl_2 \cdot 2H_2O$ in Milli Q water, pH 7.4.

Flow Cytometry—Flow cytometric analysis after co-incubation was performed as described.[2] Briefly, cells were incubated at 4 °C for 15 min in annexin V-containing BB and washed twice with 200 μl of BB. Next, the cells were taken up in 200 μl of BB, and 10 μl of 100 μM propidium iodide (PI) was added shortly before measurement. Samples were analyzed on the FACScalibur (BD PharMingen). Target cells were selected by gating the calcein-AM-positive cells. Viable cell are annexin V-allophycocyanin- and PI-negative, apoptotic cells are annexin-positive and PI-negative, and necrotic cells are annexin- and PI-positive.

Immunoblotting—Cells were lysed in TSE buffer (10 mM Tris-HCl, 250 mM sucrose, and 1 mM EGTA, pH 7.4, containing 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium vanadate, 50 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). The protein concentration in the supernatant was determined using the Bio-Rad protein assay with IgG as a standard. Twenty μg of total cellular protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). Blots were blocked with 5% (w/v) nonfat dry milk in TBS-T (0.5 M NaCl, 20 mM Tris-HCl, 0.05% (v/v) Tween 20, pH 7.4) and probed with the primary antibody. Next, blots were incubated with secondary antibody containing horseradish peroxidase and visualized with ECL reagent (Amersham Biosciences).

Mitochondrial Membrane Potential Measurements—To determine the mitochondrial membrane potential of CC531 cells during co-incubation with 2PK3 cells, 3×10^5 CC531 cells were plated on a coverglass (Knittel Gläser, Braunschweig, Germany) and cultured overnight at 37 °C in culture medium. The following day, these CC531 cells were loaded with 5 μM rhodamine-123 (Sigma-Aldrich) at 37 °C for 20 min. During the loading of the CC531s cells, the effector cells were harvested, counted, and briefly incubated with 0.2 μM rhodamine-123. The excess rhodamine was removed from the CC531 cells, and 2PK3 cells (3×10^6) were added while the coverglass was constantly kept at 37 °C

[2] J. H. L. Velthuis, H. J. G. M. de Bont, J. P. Medema, P. J. K. Kuppen, G. J. Mulder, and J. F. Nagelkerke, submitted for publication.

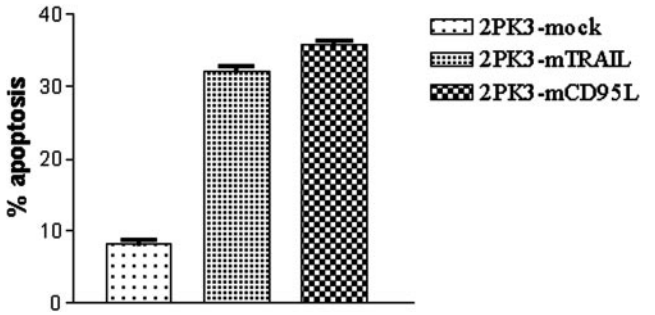

FIG. 2. **Apoptosis in CC531s cells caused by CD95L and TRAIL.** CC531s cells were co-incubated with 2PK3-mock, 2PK3-mCD95L, or 2PK3-mTRAIL cells at an effector:target ratio of 10 for 3 h. Subsequently, apoptosis was determined by flow cytometry. In all additional experiments, an effector:target ratio of 10 was used.

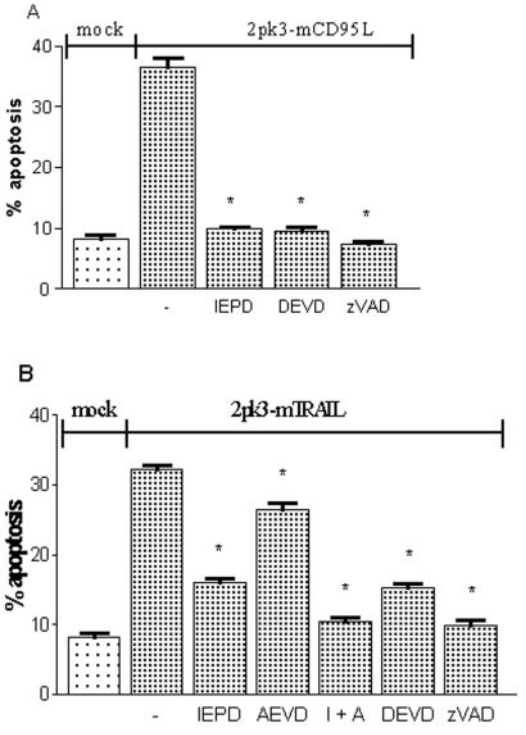

FIG. 3. **Caspase involvement in CD95L- and TRAIL-mediated apoptosis.** *A*, CC531s cells were preincubated for 30 min with the caspase inhibitors Ac-IEPD-CHO, Ac-DEVD-CHO, or zVAD-fmk. Next, they were co-incubated with 2PK3-mock or 2PK3-mCD95L cells for 3 h, and apoptosis was determined by flow cytometry. *B*, CC531s cells were preincubated with the caspase inhibitors Ac-IEPD-CHO, z-AEVD-fmk, the combination of Ac-IEPD-CHO and Ac-AEVD-CHO (*I* + *A*), Ac-DEVD-CHO, or zVAD-fmk. Next, they were co-incubated with 2PK3-mock or 2PK3-mTRAIL cells for 3 h, and apoptosis was determined by flow cytometry. *, statistically different from incubation with effector cells solely.

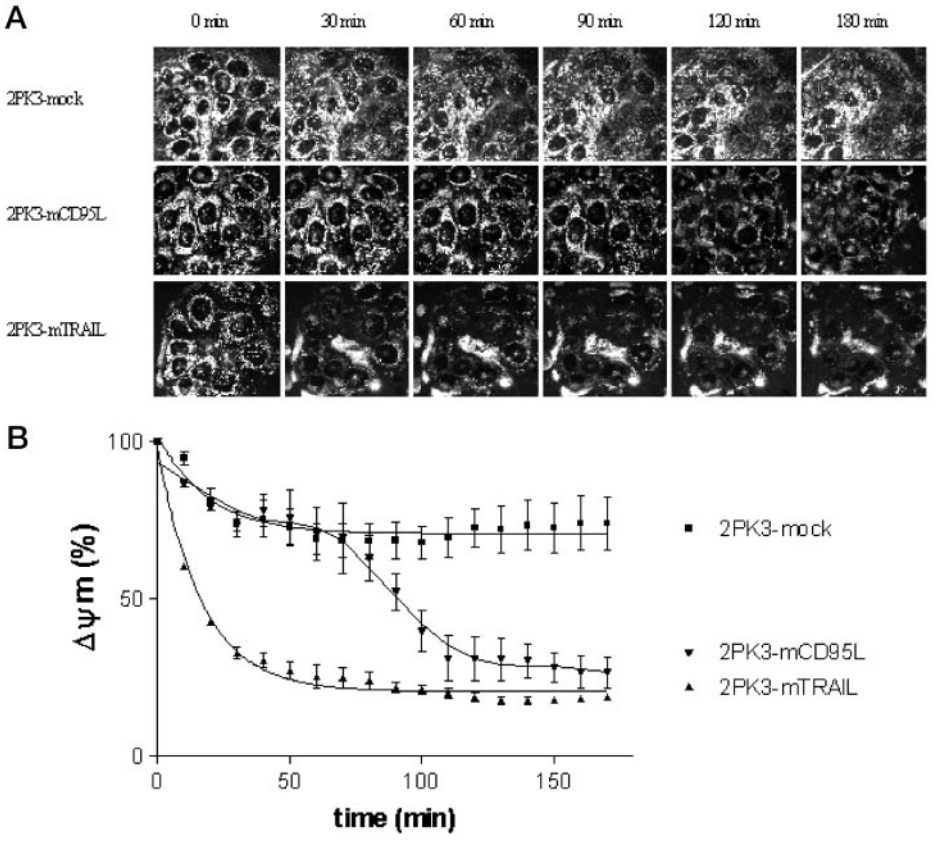

FIG. 4. **Mitochondrial membrane potential of CC531s cells after co-incubation with 2PK3 cells.** *A*, attached CC531s cells were loaded with rhodamine-123 and co-incubated for the indicated periods with 2PK3-mock, 2PK3-mCD95L, or 2PK3-mTRAIL cells. Every 2 min, a confocal laser scanning image was recorded. Several time points are shown. *B*, with 10-min intervals, recordings were quantified and plotted as the percentage of the initial value.

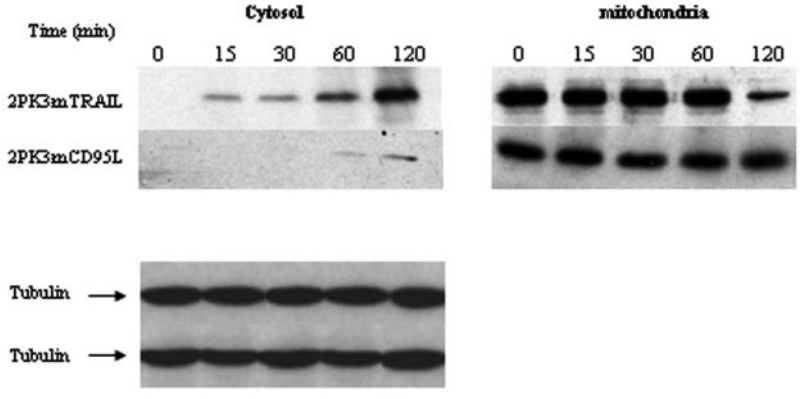

FIG. 5. **Cytochrome *c* release after TRAIL and CD95L exposure.** CC531s cells were co-incubated with 2PK3-mTRAIL or 2PK3-mCD95L cells for the indicated periods, and then the effector cells were washed off. Next, the mitochondrial and cytosolic fractions were isolated and immunoblotted for cytochrome *c*.

under a confocal laser scanning microscope. During a 3-h time course, images of rhodamine-123 fluorescence were made every 2 min.

Analysis was done by selecting a single cell and making a plot of pixel intensity *versus* the number of pixels. Herein, the mitochondria have a much higher intensity than the background. By selecting a minimal intensity, the mitochondria were separated from the background. Next, the mean pixel intensity and the number of pixels of the mitochondria were calculated and multiplied. These values are plotted as the percentage of the initial total pixel intensity *versus* time (min). At least five cells were selected and quantified per experiment.

Detection of Cytochrome c Release—CC531 cells were plated on a coverglass and allowed to attach overnight. The following day, they were co-incubated with effector cells for the indicated period. Subsequently, the cells were washed twice with phosphate-buffered saline containing 0.2% EDTA, thereby removing the effector cells but not affecting the target cells. Attached cells were analyzed by flow cytometry and proven to be >95% CC531 cells (data not shown). After separation of the two cell types, mitochondrial and cytoplasmic fractions were prepared from CC531 cells as described previously (33), and the fractions were immunoblotted for cytochrome *c*.

Statistical Analysis—Statistical analysis was performed using Student's two-sample equal variance two-tailed *t* test.

RESULTS

Receptor Expression—A prerequisite for comparing the intracellular routes leading to apoptosis after TRAIL or CD95L exposure is the presence of CD95 and all four TRAIL receptors on the target cell. Therefore, the expression of the receptors was determined on CC531s, the rat colon carcinoma cell line that we found to be sensitive toward both ligands. Immunoblots stained for CD95 and all TRAIL receptors clearly show that all receptors were present (Fig. 1). There seemed to be slightly more DR5 than DR4 at the protein level. The same was true for DcR2 as compared with DcR1. Thus, CD95 and the four TRAIL receptors were present in the CC531s cell line which made it a suitable target cell to investigate TRAIL and CD95L signaling.

TRAIL- and CD95L-induced Apoptosis in CC531s Cells—To study the effect of TRAIL and CD95L signaling, we used 2PK3 cells that had been transfected with either mTRAIL or mCD95L as effector cells, and 2PK3-mock cells served as control (34). This provided us with a tool to compare TRAIL to CD95L using the same effector and target cell line. First, we

determined TRAIL- and CD95L-induced apoptosis in CC531s cells. The cells were co-incubated with effector cells at an effector:target ratio of 10 for 3 h. Subsequently, the calcein-AM-labeled CC531s cells were stained with annexin V and PI. Using flow cytometry, cells positive for annexin V-allophycocyanin but negative for PI were identified as apoptotic. As shown in Fig. 2, 32% of the CC531s cells showed apoptotic features after co-incubation with 2PK3-mTRAIL cells, whereas co-incubation with the mock-transfected 2PK3 cell line caused no apoptosis. 2PK3-mCD95L cells as effectors resulted in 36% apoptotic CC531s cells.

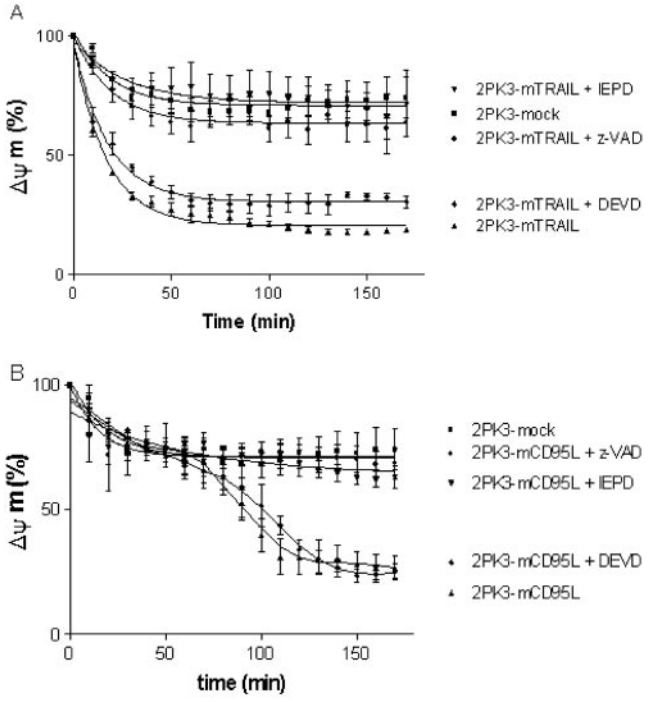

FIG. 6. **Caspase dependence of the loss of mitochondrial membrane potential of CC531s cells.** *A*, CC531s cells were loaded with rhodamine-123 and pretreated with 100 μM of the indicated caspase inhibitors. Subsequently, they were co-incubated with 2PK3-mTRAIL cells. Every 2 min, a confocal laser scanning image was recorded. With 10-min intervals, recordings of $\Delta\psi$m were quantified and plotted as the percentage of the initial value. *B,* as described in *A,* but with 2PK3-mCD95L cells as effector cells.

Involvement of Caspases—The importance of caspase-8 and -3 has been described extensively for apoptosis induced by CD95L and to a lesser extent for that induced by TRAIL. Thus, we assessed the role of upstream caspase-8 and -10 as well as downstream caspase-3 by preincubation of the CC531s cells with caspase inhibitors before the addition of the 2PK3 cells. Inhibition of caspase-8 by Ac-IEPD-CHO, inhibition of caspase-3 by Ac-DEVD-CHO, or inhibition of all caspases by zVAD-fmk completely prevented CD95L-induced apoptosis (Fig. 3*A*). In TRAIL-mediated apoptosis, inhibition of caspase-8 reduced the level of apoptotic CC531s cells from 33% to 15% (Fig. 2*B*). Addition of zVAD-fmk as well as the combined addition of Ac-IEPD-CHO and the caspase-10 inhibitor z-AEVD-fmk (indicated as $I + A$) completely prevented apoptosis. The inhibitory effect of Ac-DEVD-CHO was very similar to single addition of Ac-IEPD-CHO (the level of apoptotic cells was reduced from 33% to 15%). Addition of zAEVD-fmk only slightly reduced apoptosis by TRAIL.

Mitochondrial Dysfunction—Both TRAIL and CD95L caused a comparable level of apoptosis in CC531s cells. To find out whether the intracellular signaling pathways involved were the same, we examined whether CD95L and TRAIL signaling caused mitochondrial dysfunction. First, the effect of 2PK3-mTRAIL and 2PK3-mCD95L on the mitochondrial membrane potential ($\Delta\Psi$m) in CC531s cells was studied. Mitochondria of CC531s cells were loaded with rhodamine-123 to quantify $\Delta\Psi$m (35) before the addition of the 2PK3-mTRAIL or 2PK3-mCD95L cells. Subsequently, cells were monitored for 3 h using a confocal laser scanning microscope. Co-incubation with 2PK3-mTRAIL cells rapidly decreased the $\Delta\Psi$m of CC531s cells: after 60 min, the maximum rhodamine-123 release was almost complete (Fig. 4). Co-incubation with the 2PK3-mCD95L cell line also decreased $\Delta\Psi$m, but maximum rhodamine-123 release was not seen until ~2 h after the start of the co-incubation. The addition of 2PK3-mock cells induced no other than a spontaneous release of rhodamine-123.

Because both ligands induced loss of $\Delta\Psi$m, the release of cytochrome *c* needed for the formation of the apoptosome and activation of caspase-9 was studied. After co-incubation, the mitochondrial and cytosolic fractions of CC531s cells were im-

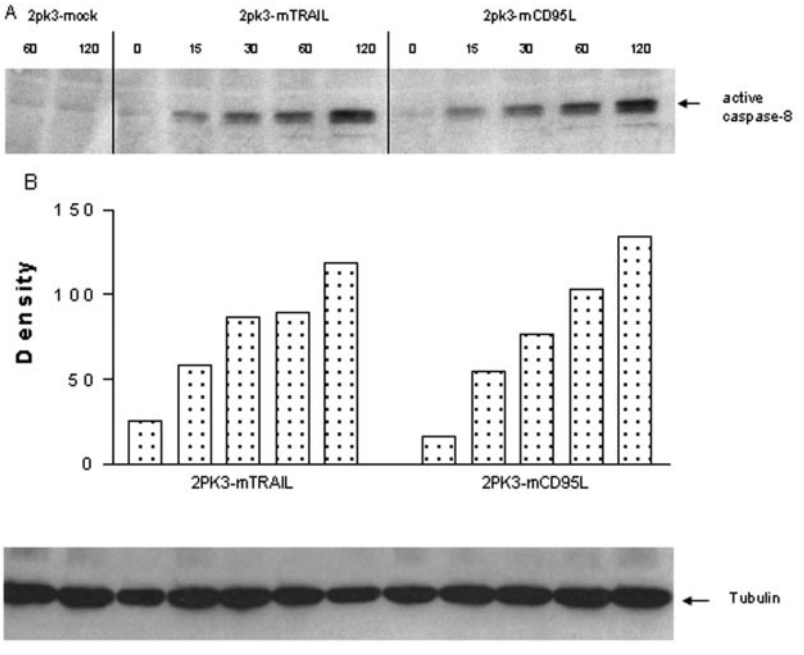

FIG. 7. **Caspase-8 activation after TRAIL and CD95L signaling.** *A*, CC531s cells were co-incubated with 2PK3-mock, 2PK3-mTRAIL, or 2PK3-mCD95L cells for the indicated periods, and then the effector cells were washed off. Next, the CC531s cells were immunoblotted for active caspase-8. *B*, immunoblot results from *A* were analyzed by densitometry.

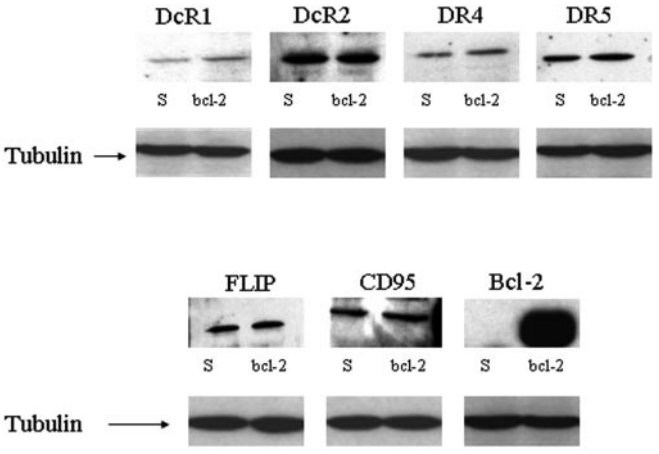

FIG. 8. **Presence of death receptors and antiapoptotic proteins in CC531s and CC531-bcl-2 cells.** CC531s and CC531-bcl-2 cells were immunoblotted for CD95, the four TRAIL receptors, FLIP, and bcl-2.

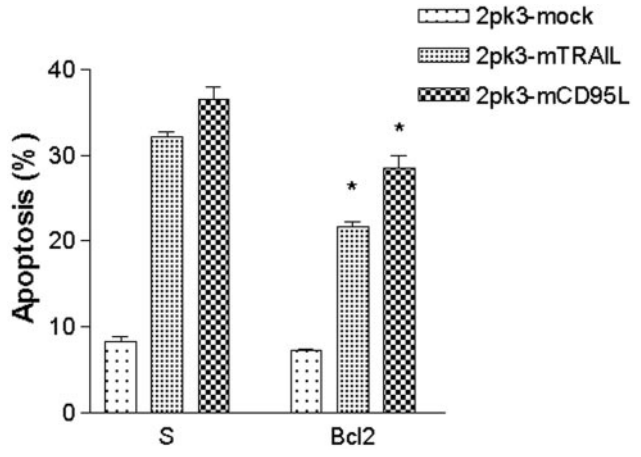

FIG. 9. **Sensitivity of CC531-bcl-2 cells toward TRAIL and CD95L.** bcl-2-overexpressing CC531 cells were co-incubated with 2PK3-mock, 2PK3-mTRAIL, and 2PK3-mCD95L cells for 3 h. Subsequently, apoptosis was determined by flow cytometry. *, statistically different from the CC531s cell line.

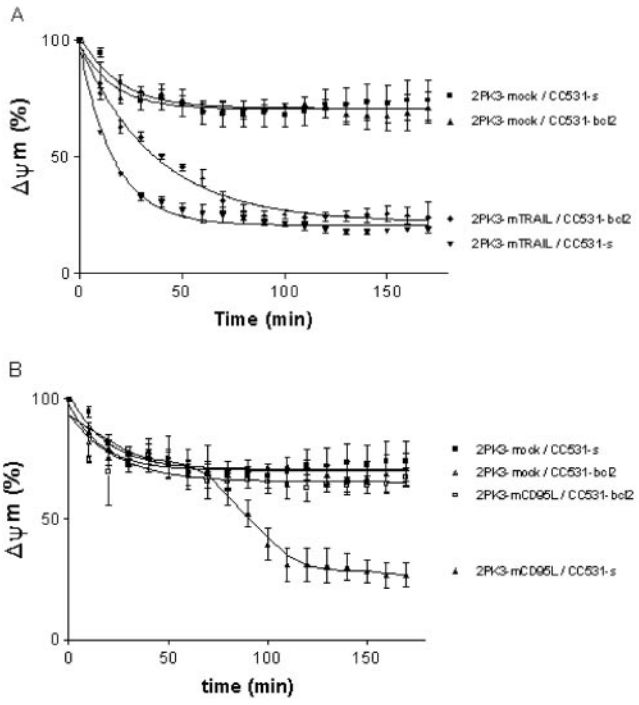

FIG. 10. **Mitochondrial membrane potential of CC531s and CC531-bcl-2 cells after TRAIL and CD95L signaling.** *A*, CC531s and CC531-bcl-2 cells were loaded with rhodamine-123 and co-incubated with 2PK3-mock and 2PK3-mTRAIL cells. Every 2 min, a confocal laser scanning image was recorded. With 10-min intervals, recordings were quantified and plotted as the percentage of the initial value. *B,* as described in *A*, but with 2PK3-mCD95L cells as effector cells.

munoblotted for cytochrome *c*. After just 15 min of 2PK3-mTRAIL exposure, cytochrome *c* appeared in the cytosolic fraction of the CC531s cells (Fig. 5), and the concentration increased with time. Consequently, in mitochondria, cytochrome *c* decreased with longer incubation periods. In contrast, the release of cytochrome *c* from mitochondria was much slower after incubation with 2PK3-mCD95L cells. Release of cytochrome *c* was only fully detectable after 120 min of exposure to the effector cells. No cytosolic cytochrome *c* was detected after co-incubation with the mock-transfected 2PK3 cells.

Caspase Involvement in Mitochondrial Dysfunction—Both TRAIL and CD95L cause mitochondrial dysfunction, although to a different extent. In the effect of both agents, an important role for caspase-8 was observed (Fig. 3); caspase-8 is capable of cleaving proapoptotic members of the bcl-2 family as well as directly activating caspase-3, which, in its turn, can cause mitochondrial dysfunction. Therefore, we studied the effects on ΔΨm in the presence of caspase inhibitors. The effect of 2PK3-mTRAIL cells could be fully prevented by addition of the caspase-8 inhibitor Ac-IEPD-CHO and the pan-caspase inhibitor zVAD-fmk (Fig. 6*A*). In contrast, the presence of the caspase-3 inhibitor Ac-DEVD-CHO had no effect on the loss of ΔΨm, indicating that loss of ΔΨm was downstream of caspase-8 but upstream of caspase-3. Addition of the caspase-10 inhibitor z-AEVD-fmk had no effect on the loss of ΔΨm (data not shown). Like TRAIL, CD95L-induced mitochondrial dysfunction was prevented by the addition of Ac-IEPD-CHO and zVAD-fmk (Fig. 6*B*). Again, inhibition of caspase-3 could not prevent the loss of ΔΨm. Therefore, CD95L-mediated loss of mitochondrial membrane potential was also a caspase-8 mediated phenomenon.

One possible explanation for the much more rapid decrease of ΔΨm after TRAIL in comparison with CD95L might be a more efficient activation of caspase-8. Therefore, CC531s cells were exposed to the effector cells for the indicated periods, lysed, and immunoblotted for active caspase-8. No difference was observed between TRAIL- and CD95L-induced activation of caspase-8 (Fig. 7*A*). Also, densitometrical analysis of the immunoblot revealed no difference (Fig. 7*B*).

Effect of bcl-2 Overexpression on TRAIL-induced Apoptosis—Because TRAIL signaling in CC531s cells caused such a strong decrease in mitochondrial membrane potential, the effect of overexpression of the antiapoptotic protein bcl-2 in a stably transfected CC531-bcl-2 cell line (32) was examined.

To confirm that bcl-2 transfection did not affect receptor expression or the level of FLIP (an inhibitor of Fas-associated death domain caspase-8 signaling), CC531s and CC531-bcl-2 cells were checked for the presence of the four receptors for TRAIL as well as CD95; the level of endogenous FLIP and bcl-2 in the CC531s and CC531-bcl-2 cells was compared by immunoblotting (Fig. 8, *S,* CC531s cells; *bcl-2,* CC531-bcl-2 cells). Clearly, the expression of the death receptors and the levels of FLIP were comparable. Apparently, the difference between the two cell lines was due only to the overexpression of bcl-2 (Fig. 8, *Bcl-2*).

Next we examined the effect of overexpression of bcl-2 in CC531 cells on TRAIL- and CD95L-mediated apoptosis (Fig. 9). TRAIL-induced apoptosis was decreased (22% *versus* 32% in CC531s cells) but not prevented. The same was seen for

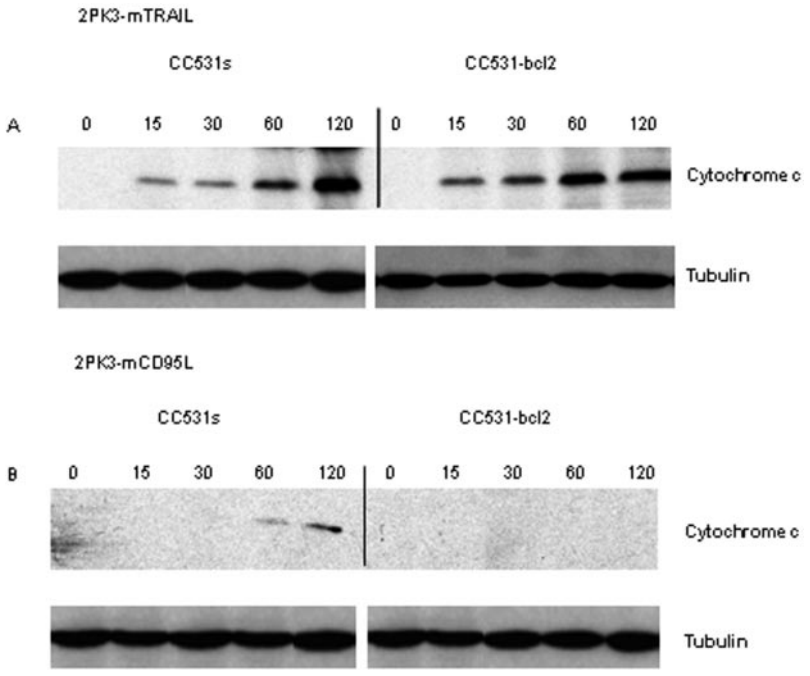

FIG. 11. **Cytochrome *c* release after TRAIL and CD95L signaling in CC531s and CC531-bcl-2 cells.** *A*, CC531s and CC531-bcl-2 cells were co-incubated with 2PK3-mTRAIL cells for the indicated periods, and then the effector cells were washed off. Next, the mitochondrial and cytosolic fractions were isolated and immunoblotted for cytochrome *c*. *B*, as described in *A*, but with 2PK3-mCD95L cells.

CD95L-mediated cell death (which decreased from 36% to 28%). Therefore, bcl-2 overexpression reduced but could not prevent apoptosis initiated by either of the two ligands.

Because bcl-2 can protect mitochondrial dysfunction after various stimuli, and TRAIL caused mainly type II apoptosis, we had expected a more pronounced effect of bcl-2 overexpression on TRAIL-induced apoptosis. Therefore, we examined the mitochondrial membrane potential of CC531-bcl-2 cells after exposure to 2PK3-mTRAIL and 2PK3-mCD95L cells. bcl-2 overexpression slightly delayed TRAIL-induced loss of $\Delta\Psi$m, but after 70 min, no difference between CC531s and CC531-bcl-2 cells was detected any more (Fig. 10*A*). In contrast, bcl-2 overexpression completely prevented CD95L-induced loss of $\Delta\Psi$m in these cells (Fig. 10*B*).

Because loss of $\Delta\Psi$m in CC531-bcl-2 cells after TRAIL signaling was little affected, the release of cytochrome *c* from bcl-2-overexpressing cells was compared with that from CC531s cells. Similar to the loss of $\Delta\Psi$m, release of cytochrome *c* was hardly affected by bcl-2 overexpression after TRAIL signaling (Fig. 11*A*). In contrast, similar to the loss of $\Delta\Psi$m, bcl-2 overexpression prevented the release of cytochrome *c* after CD95L signaling (Fig. 11*B*).

Finally, the involvement of caspases in TRAIL-mediated mitochondrial dysfunction was studied in the CC531-bcl-2 cell line. As with standard CC531 cells, the presence of Ac-IEPD-CHO or zVAD-fmk was sufficient to prevent the loss of $\Delta\Psi$m, whereas Ac-DEVD-CHO had no protective effect. Thus, in CC531-bcl-2 cells, mitochondrial dysfunction was also downstream of caspase-8 but upstream of caspase-3 (Fig. 12).

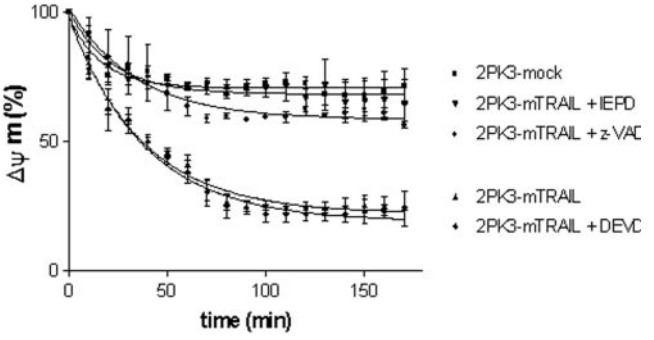

FIG. 12. **Caspase dependence of the loss of mitochondrial membrane potential of CC531-bcl-2 cells.** CC531-bcl-2 cells were loaded with rhodamine-123 and treated with 100 μM of the indicated caspase inhibitors. Subsequently, they were co-incubated with 2PK3-mTRAIL cells. Every 2 min, a confocal laser scanning image was recorded. With 10-min intervals, recordings were quantified and plotted as the percentage of the initial value.

DISCUSSION

Both TRAIL and CD95L seemed equally potent in inducing apoptosis in CC531s cells: co-incubation with 2PK3-mTRAIL or 2PK3-mCD95L cells resulted in 32% and 36% apoptotic cells, respectively. Inhibition of caspase-8 was sufficient to abolish CD95L-induced apoptosis, whereas TRAIL-mediated apoptosis was largely dependent on caspase-8, but a role for caspase-10 remained: an additional inhibition from 15% to 9% was seen. Also, inhibition of the effector caspases-3 and -7 by Ac-DEVD-CHO prevented CD95L-initiated apoptosis but only reduced TRAIL-mediated apoptosis. zVAD-fmk could completely prevent apoptosis by both ligands. These results were similar to the observations by Seol *et al.* (36), who showed that addition of z-DEVD-fmk was not sufficient to restore cell viability in the presence of TRAIL. In contrast, Kim *et al.* (10) reported that z-DEVD-fmk prevented TRAIL-induced apoptosis. Kim *et al.* (10) used Jurkat cells, whereas Seol *et al.* (36) used HeLa cells. In this respect, CC531s cells resemble HeLa cells. Furthermore, TRAIL caused such a potent disruption of the mitochondria (Fig. 4) that inhibition of the effector caspases-3 and -7 may simply be insufficient to prevent apoptosis because many mitochondrial factors are already released into the cytosol.

The much earlier occurring mitochondrial dysfunction caused by TRAIL as well as that of CD95L could be prevented by Ac-IEPD-CHO and was therefore a caspase-8-mediated effect. However, the difference in potency in mitochondrial dysfunction between TRAIL and CD95L could not be explained by a more efficient activation of caspase-8 (Fig. 7). Furthermore, overexpression of bcl-2 only slightly delayed the loss of $\Delta\Psi$m in TRAIL-mediated apoptosis as compared with CC531s cells, whereas no difference was found in release of cytochrome *c*. Possibly, a mitochondrial membrane-disrupting protein is activated by TRAIL signaling that is not inhibited by bcl-2. Apparently this disrupting protein is not activated by CD95L

signaling. These results support the previous observations by Kim *et al.* (10), who, after 16 h, no longer saw a difference between bcl-2-transfected and neo-Jurkat cells in retention of the mitochondrial dye $DiOC_6$. Our results showed a significant difference between the standard CC531s cell line and the bcl-2-transfected cell line up to 1 h after the start of the co-incubation, but not after that time point. Moreover, Keogh *et al.* (37) showed that bcl-2 overexpression completely blocked UV-induced cytochrome *c* release but failed to do so after TRAIL signaling. In contrast, both Rokhlin *et al.* (38) and Munshi *et al.* (39) reported that bcl-2 overexpression in human prostate carcinoma cell lines protected them against TRAIL-induced apoptosis. Rokhlin *et al.* (38) also reported that bcl-2 overexpression abrogated the loss of $\Delta\Psi m$ and the release of cytochrome *c*. One possible explanation might be the lack of the suggested TRAIL-activated mitochondrial disrupting protein in human prostate cancers.

Although TRAIL-induced apoptosis is strongly dependent on the mitochondrial route, it has been shown that some effects of TRAIL are mediated by direct activation of caspase-3 by upstream caspase-8 (40). Thus, TRAIL can induce apoptosis in a mitochondria-dependent and -independent manner (41), although the mitochondrial amplification loop plays an important role. Again, this is in contrast with the findings of Rokhlin *et al.* (38) and Munshi *et al.* (39) because they reported the absence of apoptosis in bcl-2-overexpressing prostate carcinoma cells, suggesting a lack of TRAIL-induced type I apoptosis in this cell type. Thus, TRAIL could be effective as a possible future treatment for several cancers (40) such as lymphoid and colon tumors, whereas for prostate cancer, it may prove to be an ineffective antitumor agent.

In CC531-bcl-2 cells, the loss of $\Delta\Psi m$ could be inhibited by the addition of Ac-IEPD-CHO. Thus caspase-8 was also responsible for the mitochondrial dysfunction in these cells. Furthermore, no delay in release of cytochrome *c* in CC531-bcl-2 cells as compared with CC531s cells was detected. In comparison, CD95L-induced cytochrome *c* release was prevented by bcl-2 overexpression, concordant with prevention of the loss of $\Delta\Psi m$ (Fig. 10*B*).

Taken together, the much more potent disruption of mitochondrial membrane potential by TRAIL as compared with CD95L and the release of cytochrome *c,* both of which were hardly inhibitable by bcl-2 overexpression but in all cases were caspase-8-mediated, indicate distinct intracellular signaling in TRAIL- and CD95L-mediated apoptosis.

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Distinct Intracellular Signaling in Tumor Necrosis Factor-related Apoptosis-inducing Ligand- and CD95 Ligand-mediated Apoptosis

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